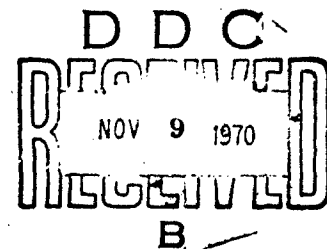UNIVERSITY OF OKLAHOMA MEDICAL CENTER

STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN FROM

GRAM-NEGATIVE BACTERIA:

CHARACTERIZATION OF THE ENZYMATICALLY DEGRADED PROTEIN MOIETY

ISOLATED BY PHENOL TREATMENT OF ENDOTOXIN FROM

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Abstract

The "simple" protein of endotoxin from <u>Serratia</u> <u>marcescens</u>
08 was treated successively with trypsin and pronase. The re-
sulting trypsin and pronase cores contained increasing amounts
of lipid A constituents firmly bound to the residual protein
moiety. Since lipid A was separated as an entity from the protein
moiety only after acid hydrolysis of the pronase core, it is
proposed that in intact endotoxin lipid A is covalently linked
to the protein moiety. The absence of any detectable glucosamine
and fatty acids in the mixture of peptides and amino acids released
by trypsin and pronase, indicated a single point attachment between
the lipid A and protein moieties. Both trypsin and pronase cores
were immunogenic and revealed the presence of a common antigenic
determinant with the parent "simple" protein. The second set of
antigenic determinant(s) different from the specific O-antigens
is located in the protein moiety close to lipid A.

Although it is recognized that the protein moiety constitutes

an integral part of the structure of undegraded endotoxin prep-

arations, its site and mode of attachment to the lipopolysaccharide

moiety is unknown (1, 2). It has been established in the preceding

paper of this series (3) that the so-called "simple" proteins

isolated by phenol treatment of endotoxins from S. marcescens 08

and E. coli O 141:K85(B) contain also lipid A constituents such

as glucosamine, phosphorus and β-hydroxy myristic acid as the

major fatty acid. Since these compounds were not separable from

the protein moiety by solvent extraction or agarose electrophoresis,

it was concluded that at least a portion of lipid A is firmly

bound to the protein moiety. To explore further the mode of

linkage between the protein moiety and lipid A constituents,

"simple" protein from S. marcescens 08 was treated successively

by proteolytic enzymes. The resulting tryptic and pronase cores

contained increasing amounts of lipid A constituents firmly

bound to the residual protein. Since lipid A was removed only

after acid hydrolysis of cores, we propose that the protein moiety

is covalently linked to lipid A.

MATERIALS AND METHODS

Enzymes

Trypsin was a 2 X crystallized preparation treated with

diisopropyl phosphofluoridate (DFP) and was purchased from

Worthington (Freehold, N. J.). Pronase, B grade, from Streptomyces

griseus was obtained from Calbiochem, Los Angeles, Calif. DFP-treated

Carboxypeptidase A was purchased from Worthington, Freehold, N. J.

Isolation of "Simple" Proteins

"Simple" proteins of endotoxins from S. marcescens 08 (PX-S)

and E. coli O 141:K85(B) (PX-E) were isolated according to a

procedure previously described (3).

Preparation of the Tryptic and

Pronase Cores of PX-S

Fifty milligrams of PX-S were suspended in 15 ml 0.02 M

ammonium carbonate buffer, pH 8.6, heated for 30 seconds in a

boiling water bath and cooled in an ice bath to 37°. Trypsin,

dissolved in a small volume of the same buffer, was added to the

suspended protein (trypsin/protein, 1:100, w/w) and the reaction

mixture was stirred gently for 12 hours at 37° (digestion was

completed after 8-10 hours, as determined photometrically by the

ninhydrin reaction). After hydrolysis the clear reaction mixture

was heated again for 30 seconds in a boiling water bath, cooled

to room temperature and acidified with acetic acid (pH 3.5-3.8).

The precipitate which formed upon standing overnight at 4° was

removed by filtration through a membrane filter AM7 (Gelman

Instrument Co., Chelsea, Michigan) or by centrifugation at 12,000

rpm (17,300 x g) for 20 minutes at 10°. The sediment was redissolved

in ammonium carbonate buffer and precipitated again at pH 3.5-3.8

with acetic acid. After washing 3X with acetic acid and 2X with

distilled water, the precipitate was lyophilized and designated

tryptic core of PX-S. The combined, lyophilized supernates

represented the tryptic peptides.

The tryptic core of PX-S was digested with pronase according

to a similar procedure; since maximum release of amino acids was

reached after 20-21 hours, the hydrolysis time was prolonged to

24 hours. The corresponding fractions were called pronase core

of PX-S and pronase peptides. The tryptic and pronase cores were

tested by high voltage electrophoresis for the possible presence

of ninhydrin-positive impurities. If necessary, the cores were

washed with distilled water until free of impurities.

Preparation of Carboxypeptidase Core of PX-S

Two milligrams of pronase core of PX-S were dissolved in a

small volume of 0.05 M Tris buffer containing 0.5% sodium dodecyl

sulfate (SDS), pH 7.6. To this solution 0.2 M N-ethyl-morpholine

buffer, pH 8.5, was added to a final volume of 1 ml. The final

concentration of SDS was 0.2%. Carboxypeptidase A was added in

a substrate/enzyme ratio 80:1 and the mixture was incubated at 37°.

Aliquots were taken at different time intervals within 72 hours

and the undigested material was precipitated with 0.1 N HCl. The

combined supernates, evaporated to dryness and dissolved in

citrate buffer pH 2.2, were used for the amino acid analysis.

The final precipitate, washed with distilled water and lyophilized,

represented the carboxypeptidase core of PX-S.

Isolation of Lipid A from "Simple" Protein

of S. marcescens 08

PX-S was added to preheated 0.1 N HCl (mg PX-S/0.2 ml acid)

and hydrolyzed for 30 minutes at 85-90°. After cooling in an ice

bath, the hydrolysate was centrifuged at 12,000 rpm (17,300 x g)

for 10 minutes at 4°. The supernate and the remaining pellet

were separately extracted 3X by chloroform. The combined chloroform

extracts from both supernate and pellet were washed exhaustively

with deionized water to remove traces of acid and evaporated to

dryness under reduced pressure. The lipid residue was treated

several times with boiling acetone. The acetone insoluble

material (lipid A) was removed by filtration. The unhydrolyzed

-pellet (protein moiety) was washed with ice-cold water and lyophilized.

Electrophoresis and Infrared Spectroscopy

Agarose electrophoresis was carried out in 1% agarose according to the procedure by Grabar and Williams (4) employing Veronal buffer, pH 8.6, ionic strength 0.05, for 40 minutes. Protein samples (20 mg/ml) were dissolved in 0.05 M Tris buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in Veronal buffer.

The infrared spectra of PX-S, tryptic and pronase cores and lipid A were obtained with a Beckman Infrared Spectrophotometer JR10 using potassium bromide pellets (1.0-1.8 mg of substance and 250 mg KBr).

Analytical Methods

Procedures for the quantitative analysis of amino acids, fatty acids, glucosamine, phosphorus, nitrogen, carbon, hydrogen and ash were described in a previous paper (3). Sephadex G-50 was used for the gel filtration column chromatography of the pronase core of PX-S.

Immunological Methods

Preparation of antibodies to PX-S and tryptic and pronase

cores and double diffusion experiments in 1% agar gel were performed

according to the procedures previously described (3). Antibodies

against an essentially protein-free lipopolysaccharide (LPS-A)

from S. marcescens 08 were prepared by immunizing white rabbits

with four successive intraperitoneal injections of 0.5 mg of antigen

dissolved in 2 ml 0.05 M Tris-HCl buffer containing 0.5% SDS and

emulsified with 2 ml of Freund's complete adjuvant. The injections

were administered at weekly intervals, and antibodies were detected

after the fourth injection. Even a slight increase in dosage

of LPS-A caused death of the animals.

Antigens and the homologous antibodies are symbolized by the

same letters; capital letters designate antigens and small letters

antibodies:

Antigens		Antibodies	
PX-S	A	Anti PX-S	a
Tryptic core	E	Anti tryptic core	e
Pronase core	F	Anti pronase core	f
LPS-A	M	Anti LPS-A	m

RESULTS

Preparation of Tryptic and Pronase Cores of PX-S

Tryptic core of PX-S, isolated by isoelectric precipitation

at pH 3.5-3.8, accounted for 64.5% of PX-S. The purple color of

the tryptic core indicated that prodigiosin or one of its

derivatives was still firmly attached to this portion of the

protein moiety. The corresponding peptide fraction was colorless.

The purple-colored pronase core, precipitated at the same pH range

(3.5-3.8), accounted for 28.7% of the tryptic core and for 18.5%

of PX-S. To remove an additional amount of protein moiety, pronase

core was treated successively with carboxypeptidase A; carboxy-

peptidase A released amino acids, which were in very small amount.

Tryptic and pronase digestion of "simple" protein from $\underline{E}$. $\underline{coli}$ (PX-E) resulted in the isolation of a tryptic core (60.3% of PX-E) and a pronase core (20.4% of PX-E); both cores were isolated by isoelectric precipitation at pH 3.5-3.8.

Physical-chemical Characterization of the Tryptic

and Pronase Cores of PX-S

Both cores were readily soluble in 0.02 M ammonium carbonate buffer, pH 8.6, but were insoluble at a pH lower than 3.8, in water or in saline. However, after repeated lyophilizations, tryptic and pronase cores were soluble only if the ammonium carbonate buffer contained 0.1-0.2% SDS.

. On agarose electrophoresis, tryptic and pronase cores migrated as single bands with mobilities similar to that of PX-S (Fig. 1). Gel filtration of pronase core on Sephadex G-50 yielded a single fraction characterized by a symmetrical elution curve with absorption maximum at 280 mμ. The amino acid and glucosamine analyses showed the same values before and after column chromatography.

The infrared spectra of the tryptic and pronase cores were similar to that of PX-S (Fig. 2). Proteolytic shortening of the protein moiety (PX-S→pronase core) resulted in a decrease in the ν OH ass. and the ν NH ass. absorption at 3400–3280 cm^{-1}, and an increase in the ν CH_3 + CH_2 absorption at 2960–2850 cm^{-1} and the carbohydrate absorption at approximately 1060 cm^{-1} (γ OH and ν C-O). The most striking feature in the spectrum of the pronase core was the appearance of a strong ester absorption ν C = O at 1740 cm^{-1} which could be recognized in the spectra of PX-S and tryptic core only as a shoulder on the amide I absorption band at 1650 cm^{-1}.

The pronounced ester absorption in the spectrum of the pronase core was obviously caused by a decreased protein and increased fatty acid ester content in accordance with the simultaneous increase of ν CH_3 + CH_2 absorption at 2960–2850 cm^{-1} as well as with the results of chemical analysis.

Immunological Properties of Tryptic and Pronase Cores of PX-S

Tryptic core (E) and pronase core (F) showed single, identical

precipitin lines with antibodies to PX-S (a) and exhibited identity

with PX-S (A) (Fig. 3, pattern 1). In immunoelectrophoresis both

cores gave single precipitin arcs with antibodies to PX-S. Tryptic

and pronase cores were immunogenic. PX-S (A), tryptic core (E) and

pronase core (F) showed complete coalescence of precipitin lines

with antibodies to tryptic core (Fig. 3, pattern 2) and with anti-

bodies to pronase core (Fig. 3, pattern 3). Since both cores were

immunogenic, the antigenic determinant(s) may be located either on

the residual protein fragment close to lipid A or on the lipid A

moiety itself. To resolve this alternative, a lipopolysaccharide

preparation (LPS-A) containing less than 2% amino acids and PX-S

were tested with antibodies to LPS-A (m) and/or antibodies to PX-S (a).

Since LPS-A consists of a polysaccharide moiety and lipid A, and

PX-S of a protein moiety and lipid A, a reaction of partial identity

would indicate the presence of a second set of antigenic determinants

within the lipid A. In contrast, a non-identity reaction would

indicate the occurrence of antigenic determinant(s) in the protein

moiety. Results of these experiments showed that neither LPS-A (M)

reacted with antibodies to PX-S (a) nor PX-S (A) with antibodies

to LPS-A (m) (Fig. 4, left pattern). Moreover, PX-S (A) and

LPS-A (M) showed a non-identity reaction with a mixture of antibodies

to PX-S (a) and LPS-A (m) (Fig. 4, right pattern). Thus, the second

set of antigenic determinants in the endotoxin complex is located

in the protein rather than the lipid moiety.

Chemical Characterization of the Tryptic and

Pronase Cores of PX-S

Results of the elementary analysis and chemical composition of

the tryptic and pronase cores are compared with those of PX-S

(Table 1). Shortening of the protein moiety (PX-S→pronase core)

caused an increase in the relative content of carbon, hydrogen,

phosphate, glucosamine and fatty acids and a decreased content of

nitrogen and amino acids. Proteolysis of the tryptic core by

pronase caused a more drastic compositional change than that

caused by trypsin treatment of PX-S. This change was also clearly

reflected in and paralleled by similar changes in the infrared

spectra and yields of tryptic (64% of PX-S) and pronase (18% of

PX-S) cores. PX-S as well as its cores contained the characteristic

components of lipid A with an approximate molar ratio of fatty

acids/phosphate/glucosamine of 2:1:1. The relatively low recovery

of the constituents of the pronase core (60.6%) may be explained

by a loss of amino acids known to occur when hydrolysis is

carried out in the presence of carbohydrates (5), and by the fact

that the absolute amount of prodigiosin, present in the pronase

core in the highest concentration, is unknown.

Results of the amino acid analysis of tryptic and pronase

cores and their corresponding peptide fractions are shown in Table 2.

Peptide fractions contained no detectable glucosamine or fatty acids.

Aspartic acid was the major amino acid of all three cores. The

relative content of glucosamine and the molar ratios of aspartic

acid and glucosamine of PX-S and corresponding cores are shown

in Table 3. From the molar ratios of glucosamine and major amino

acids of pronase and carboxypeptidase cores (Table 4), it was

concluded that aspartic acid, glutamic acid and serine represented

the amino acids most likely to be involved with glucosamine in a

covalent linkage between the lipid A and protein moiety.

The fatty acid composition of PX-S and its cores was character-

ized by the presence of β-hydroxy myristic acid, the typical marker

for lipid A, in a concentration higher than any other long-chain

saturated or unsaturated fatty acid (Table 5).

Isolation of Lipid A from "Simple" Protein

Although chemical analysis of PX-S and its cores indicated

clearly the presence of lipid A constituents, an intact lipid A

moiety could not be separated from the protein moiety by electro-

phoresis or by exhaustive extraction with chloroform. However,

when PX-S was hydrolyzed with 0.1 N HCl according to a standardized

procedure used for the isolation of lipid A from lipopolysaccharide,

a chloroform soluble but acetone insoluble fraction was obtained.

The infrared spectrum (Fig. 2) of this fraction was identical

with the spectrum of lipid A isolated from the lipopolysaccharide

fraction (LPS-A) and was very similar to that of the pronase core.

The slightly higher ester/amide I ratio (0.86) of this lipid

fraction in comparison with that (0.79) of pronase core could be

explained by its decreased protein content and, therefore, a

decreased amide absorption. The glucosamine content of this

preparation was 27.7%. These results indicated that this fraction

identified as lipid A is very likely covalently linked to the

protein moiety.

DISCUSSION

The "simple" proteins PX-S and PX-E were only partially degraded

by successive digestion with trypsin and pronase. A similar, partial

degradation of the protein moiety by tryptic digestion has also

been reported for the endotoxin complex of Salmonella typhosa (6, 7),

Shigella paradysenteriae (8, 9), E. coli (10) and Neisseria

gonorrhoeae (11). On the other hand, Morgan and Partridge (12)

claimed that tryptic hydrolysis of endotoxin from Shigella

dysenteriae resulted in an almost complete removal of the protein

moiety. It is not known whether the partial tryptic hydrolysis

of the protein is due to a particular amino acid sequence or to

an inhibitory effect of the protein-bound lipid A on the enzymatic

activity. However, even pronase digestion of the tryptic core of

PX-S resulted in an incomplete removal of the protein moiety.

Recently, Rosselet et al., (13) isolated from E. coli a proteinaceous

component by phenol-water treatment of bacterial extracts. This

phenol-soluble fraction, called "protodyne," contained 87.7%

protein, 1.2% carbohydrate, 0.3% amino sugars and 0.9% lipid and

exhibited the ability to enhance nonspecific host resistance to

infection. Upon pronase digestion, the remaining residue contained

still an appreciable amount of protein (62.9%) but also an increased

amount of lipid (8.6%). Since neither of these two substances

contained 2-keto-3-deoxy-octonate (KDO), the authors concluded

that "protodyne" contained no lipopolysaccharide and therefore

differed from this cell wall constituent. However, this does not

exclude the possibility that "protodyne" represents the protein

moiety of endotoxin, because KDO, linking lipid A with the

polysaccharide moiety, is present in the water soluble lipopoly-

saccharide and not in the phenol-soluble protein moiety. It

seems, therefore, that "protodyne" and its pronase core may be

similar to "simple" proteins PX-S and PX-E and the corresponding

pronase cores. Pronase digestion of both "protodyne" and PX-S

preparations resulted in an incomplete removal of proteins.

Although the successive enzymatic degradation of "simple"

proteins resulted in only partial removal of protein moieties,

the tryptic and pronase cores contained increasing amounts of

lipid A constituents which could not be separated from the remaining

protein residue by isoelectric precipitation, gel filtration or

by extraction with chloroform. Since lipid A constituents could

be isolated as an entity only after hydrolysis of the pronase

core of PX-S with 0.1 N HCl, we suggest that,at least in the intact

endotoxin of S. marcescens 08, lipid A is covalently linked to

the protein moiety. In contrast to the more usual random attachment

of carbohydrate chains along the polypeptide backbones of several

structurally known glycoproteins (14), we propose a single point
of linkage between lipid A and protein. This proposal is based
on the following evidence: 1) peptides obtained by enzymatic
digestion of PX-S with trypsin, pronase and carboxypeptidase A
were free of glucosamine and fatty acids, and 2) the glucosamine/
aspartic acid ratio of the carboxypeptidase core (10:1) was twice
as high as that of the pronase core (5:1). The amino acid analysis
of the carboxypeptidase core indicated aspartic acid, glutamic
acid and serine as amino acids most likely to be linked with the
terminal glucosamine unit of lipid A.

Antibodies prepared against "simple" protein and its tryptic
and pronase cores revealed the presence of a common antigenic
determinant(s) different from the determinants of the O-specific
side chain of the polysaccharide moiety. Homma and his coworkers
(15, 16) have demonstrated that endotoxins isolated from the
autolysates of Pseudomonas aeruginosa contain antigenic determinants
in the polysaccharide as well as in the protein moiety. Recently,

Maeland (17, 18) has established that endotoxin preparations

obtained by either aqueous-ether or trichloroacetic acid extraction

of Neisseria gonorrhoeae contain also two antigenic determinants,

one of which (determinant a) is located in the polysaccharide

and the other (determinant b) in the protein moiety. Like fraction

LPS-A in this study, the lipopolysaccharide fragment isolated by

phenol-water extraction contained only determinant a (O-specific

antigen). The determinant located in the protein moiety of Neisseria

gonorrhoeae was destroyed by pronase, but not by trypsin digestion.

Since in the present study the pronase core of "simple" protein

PX-S was still immunogenic, the antigenic site is most probably

positioned close to the lipid A moiety.

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Table 1

Elementary Analysis and Chemical Composition of PX-S, Tryptic Core and Pronase Core

	C %	H %	N %	PO_4 %	Glucosamine %	Fatty Acids %	Amino Acids %	Recovery[a] %	Ash %
PX-S	51.2	6.9	13.4	2.0	2.9	9.2	72.9	87.0	4.9
PX-S Tryptic core	51.3	7.1	11.1	3.1	4.8	11.4	69.0	88.3	4.3
PX-S Pronase core	52.3	8.0	4.0	5.8	12.1	31.3	11.5	60.7	3.6

[a] Recovery was calculated from phosphate, glucosamine, fatty acid and amino acids.

Table 2

Amino Acid Composition of PX-S, Tryptic Core, Pronase Core, and Peptides

	PX-S		Tryptic peptides		Tryptic core		Pronase peptides		Pronase core	
	µMoles/g	mg/100	µMoles/g	mg/100	µMoles/g	mg/100	µMoles/g	mg/100	µMoles/g	mg/100
Lysine	245	3.58	246	3.60	142	2.08	165	2.41	52	0.76
Histidine	74	1.15	56	0.87	80	1.24	75	1.16	13	0.20
Arginine	181	3.15	156	2.72	95	1.65	117	2.04	27	0.47
Aspartic acid	824	10.97	811	10.79	876	11.66	1640	21.83	142	1.89
Threonine	422	5.03	394	4.69	402	4.79	570	6.78	50	0.60
Serine	442	4.64	360	3.78	490	5.15	610	6.41	122	1.28
Glutamic acid	537	7.90	471	6.93	477	7.02	632	9.30	69	1.02
Proline	137	1.58	130	1.50	219	2.52	223	2.57	31	0.36
Glycine	657	4.93	598	4.49	600	4.50	867	6.51	89	0.67
Alanine	657	5.85	657	5.85	680	6.05	862	7.68	109	0.97
1/2 Cystine	-	-	-	-	-	-	-	-	-	-
Valine	378	4.43	419	4.91	307	3.60	482	5.65	60	0.70
Methionine	42	0.63	57	0.85	60	0.90	89	1.33	15	0.22
Isoleucine	238	3.12	227	2.98	182	2.39	268	3.52	45	0.59
Leucine	478	6.27	385	5.05	535	7.02	695	9.12	66	0.87
Tyrosine	240	4.35	222	4.02	235	4.26	350	6.34	17	0.31
Phenylalanine	228	3.77	188	3.11	235	3.88	322	5.32	32	0.53

Table 3

Percent Content of Glucosamine and Molar Ratios of Aspartic Acid/Glucosamine in PX-S and Its Cores

	Glucosamine in %	Molar Ratio Aspartic Acid:Glucosamine
PX-S	2.87	6:1
Tryptic core	4.81	3:1
Pronase core	12.09	1:5
Carboxypeptidase core	16.12	1:10

Table 4

Molar Ratio of Glucosamine and Major Amino Acids in the

Pronase and Carboxypeptidase Cores of PX-S

	Pronase Core	Carboxypeptidase Core
Glucosamine	10	10
Aspartic acid	2	1
Serine	2	1
Glycine	1	1
Alanine	2	1
Glutamic acid	1	1

Table 5

Percent Fatty Acid Composition of PX-S, Tryptic Core

and Pronase Core

	PX-S %	Tryptic core %	Pronase core %
C_{12}	2.6	3.7	3.4
C_{14}	9.5	9.8	10.7
C_{14-1}	Trace	0.0	0.0
Unknown	12.9	14.6	14.4
C_{16}	16.5	18.2	13.3
C_{16-1}	5.5	3.4	2.8
Unknown	Trace	3.1	5.7
Unknown	3.9	4.7	2.0
Unknown	4.5	3.7	5.8
C_{14-OH}	44.5	38.7	41.8
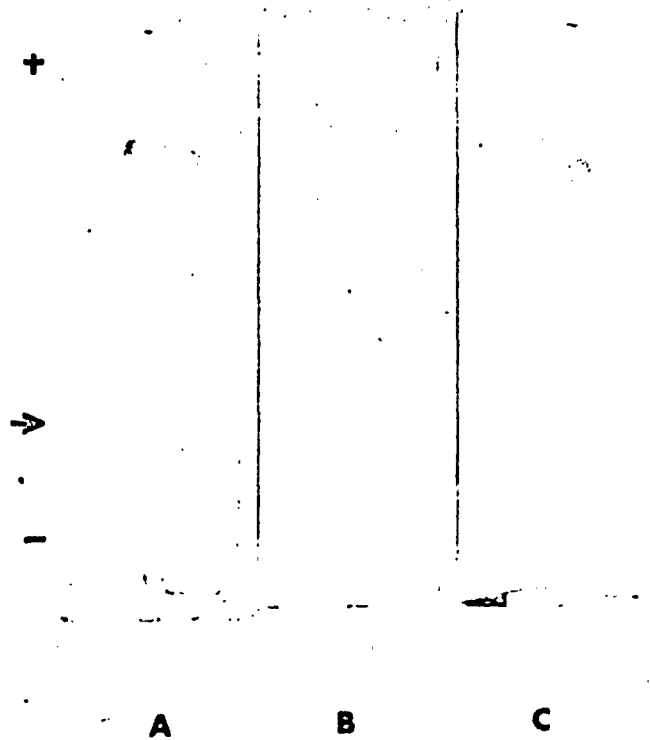
Figure 1. Agarose electrophoresis of "simple" protein PX-S (A).
tryptic core (B) and pronase core (C). Protein preparations
(20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer, pH 7.6,
containing 0.5% SDS, and diluted to a final concentration of
10 mg/ml with 1% agarose in Veronal buffer. Electrophoresis
was carried out for 40 minutes in 1% agarose gel employing
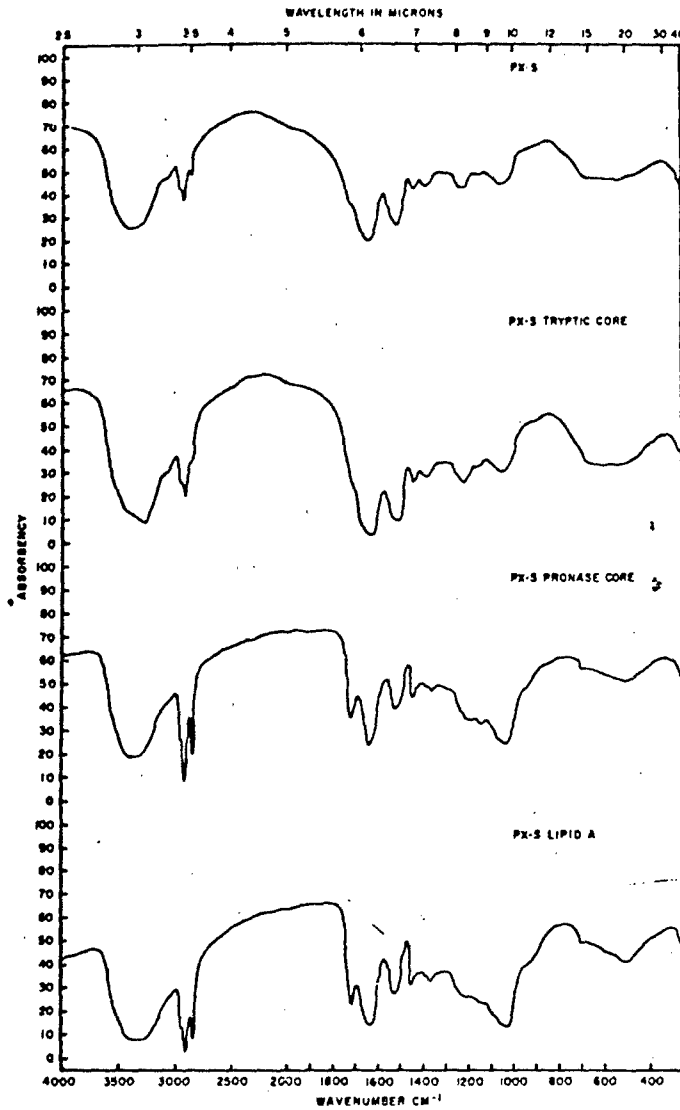Veronal buffer, pH 8.6, ionic strength 0.05.

Figure 2. Solid infrared spectra of "simple" protein PX-S and its
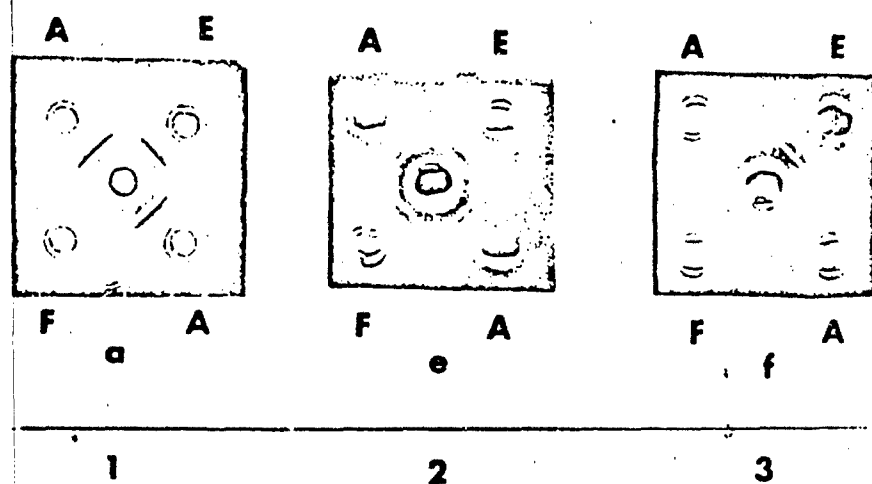corresponding tryptic core, pronase core and lipid A.

Figure 3. Immunodiffusion patterns of "simple" protein PX-S (A), tryptic core (E) and pronase core (F). Central wells contain antibodies to PX-S (a), tryptic core (e) and pronase core (f).
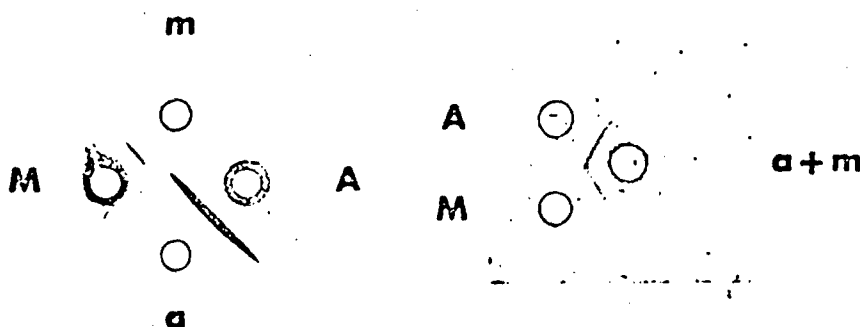
Figure 4. Immunodiffusion patterns of PX-S (A) and LPS-A (M).
Antibodies to PX-S are present in well a and antibodies
to LPS-A in well m. Well a + m contains a mixture of
equal amounts of antibodies to PX-S and LPS-A.

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13. ABSTRACT

The "simple" protein of endotoxin from Serratia marcescens 08 was treated successively with trypsin and pronase. The resulting trypsin and pronase cores contained increasing amounts of lipid A constituents firmly bound to the residual protein moiety. Since lipid A was separated as an entity from the protein moiety only after acid hydrolysis of the pronase core, it is proposed that in intact endotoxin lipid A is covalently linked to the protein moiety. The absence of any detectable glucosamine and fatty acids in the mixture of peptides and amino acids released by trypsin and pronase, indicated a single point attachment between the lipid A and protein moieties. Both trypsin and pronase cores were immunogenic and revealed the presence of a common antigenic determinant with the parent "simple" protein. The second set of antigenic determinant(s) different from the specific O-antigens is located in the protein moiety close to lipid A.

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